Differences in Epitope Restriction of Autoantibodies to Native Human Insulin (IAA) and Antibodies to Heterologous Insulin (IA)

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SUMMARY

The binding profiles of insulin autoantibodies (IAA) and insulin antibodies (IA) to highly purified species variants and fragments of insulin were studied in direct immunospecific enzyme-linked immunosorbent assay (ELISA) and indirect absorption experiments with insulins covalently coupled to Sepharose beads. Five of 10 IAA-containing sera from insulin-naive nondiabetics that bound whole human (H) insulin did not bind whole porcine (P) or whole bovine (B) insulins. These sera bound H insulin B-chain but not P B-chain or desalanated P insulin, suggesting they were dependent on the presence of threonine B30. The other 5 IAA-containing sera bound H, P, B, and desalanated porcine insulins, but only 1 bound isolated B-chains. All 10 IA-containing sera from insulin-treated diabetics bound H, P, B, and desalanated P insulins, but only 1 bound to human (and porcine) B-chain. Further binding studies with ovine, rabbit, rat, and guinea pig insulins confirmed the H (threonine B30) specificity of the 5 IAA-containing sera. B30 residues do not appear to be dominant, however, when insulin is administered exogenously. Instead, IA bind predominantly to A-chain or conformational determinants involving both chains. Scatchard analysis of a representative H insulin–specific IAA serum suggested that it contained a single binding affinity, whereas analysis of a representative IA-diabetic serum suggested it contained several different affinities. *Diabetes* 36:66–72, 1987

Insulin antibodies (IA) are commonly found in the sera of insulin-treated diabetics and occasionally cause the clinical symptoms of insulin resistance (1), allergy (2), or lipoatrophy (3). Such sera have been viewed as polyclonal and unable to discriminate between the largely homologous amino acid sequences of the bovine (B), porcine (P), and human (H) insulins used in clinical practice. Studies on closely inbred animals, however, suggest that individual strains respond immunologically to different epitopes on the insulin molecule, sometimes to the extent of mutual exclusion (4,5).

Insulin autoantibodies (IAA) were first reported some 15 yr ago in the context of the autoimmune hypoglycemia syndrome (6). Recently, we described IAA-containing sera specific for H insulin that failed to bind either P or B insulin (7). Because H and P insulin differ by a single amino acid at B30 (threonine in H and alanine in P insulin), IAs in these sera were, by implication, directed exclusively toward the threonine B30 residue of H insulin or some epitope dependent on it. Other IAA-containing sera did not distinguish H from P and B insulins, but in view of the large number of epitopes shared by the three insulins it was not clear whether the pattern of antigen restriction in these sera would be the same as that in the IA-containing diabetic sera. The aim of this study is to establish the patterns of antigen restriction of IA and IAA by observing their binding profiles to an extensive panel of insulin fragments and species variants.

MATERIALS AND METHODS

Sera. Twenty sera from two groups of patients were studied. The first 10 were IAA-positive sera from patients (5 male, 5 female; age range 26–67 yr, mean 46.1 yr) referred to the Autoimmune Profile Laboratory with provisional diagnosis of an autoimmune disorder. They were selected from 64 IAA-positive sera identified from 2000 received by the laboratory (frequency 3.2%). Five of the 10 sera tested were drawn from 39 (61%) of the original 64 found to be specific for H insulin (Nos. 1–5), and 5 were from the remaining 25 (39%) that bound H, P, and B insulins equally (Nos. 6–10). None of the patients came from the same family grouping, none had diabetes or a family history of diabetes, none had a history of hypo- or hyperglycemia, and none had received

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TABLE 1
Binding profiles of 10 IAA-containing sera (Nos. 1–10) and 10 IA-containing diabetic sera (Nos. 11–20) with different insulin species variants and modified insulins in direct binding ELISA

Serum no.	Insulin antibodies									
	Human	Porcine	Bovine	Human B-chain	Porcine B-chain	Desalanated porcine	Rabbit	Ovine	Rat	Guinea pig
1	+	–	–	+	–	–	–	–	–	–
2	+	–	–	+	–	–	–	–	–	–
3	+	–	–	+	–	–	–	–	–	–
4	+	–	–	+	–	–	–	–	–	–
5	+	–	–	+	–	–	–	–	–	–
6	+	+	+	–	–	+	+	+	–	–
7	+	+	+	–	–	+	+	+	–	–
8	+	+	+	–	–	+	+	+	+	+
9	+	+	+	–	–	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	–
11	+	+	+	–	–	+	+	+	–	–
12	+	+	+	–	–	+	+	+	+	–
13	+	+	+	–	–	+	+	+	–	+
14	+	+	+	–	–	+	+	+	–	–
15	+	+	+	–	–	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	–
17	+	+	+	–	–	+	+	+	–	–
18	+	+	+	–	–	+	+	+	–	–
19	+	+	+	–	–	+	+	+	–	–
20	+	+	+	–	–	+	+	+	–	–

insulin. Insulin naivety was obtained in every case by letter from the patient, the general practitioner, and the hospital consultant or case notes. No correlation was demonstrated between the presence or specificity of IAA and the presence or specificity of 15 other autoantibodies routinely measured by the laboratory, as noted previously (7).

The second group of sera (Nos. 11–20) were obtained from 10 insulin-dependent diabetics (4 male, 6 female; age range 40–80 yr, mean 56.2 yr). All had been controlled with B or P insulin for 13–35 yr (mean 20.6 yr). One hundred control sera were obtained from the hospital's blood transfusion service.

Assay methods. The IA content of sera was measured against highly purified insulin species variants and fragments of insulin (courtesy of Dr. Mary Root, Lilly, Indianapolis, IN) by a direct micro enzyme-linked immunosorbent assay (ELISA) method specific for human IgG (8), in which the insulins were coated onto polystyrene plates at a concentration of 0.5 μg/well and the sera assayed at a dilution of 1:30. The antigens used were whole H, P, B, rabbit, ovine, rat, and guinea pig insulins, H B-chain, P B-chain, and Desalanated P insulin (B30 deleted). The isolated P B-chain was obtained by cleavage, and the H B-chain was produced biosynthetically.

The results were derived from a reference curve for the appropriate species of insulin, obtained by the serial dilution of a single reference serum incorporated into every microtiter plate. The reference serum consisted of pooled sera from 5 diabetics in order to obtain a broad representation of binding sites. Results for each serum were calculated from the reference curve against the relevant antigen, in terms of insulin-binding units. For the sake of clarity, however, the results in Table 1 have been given simply as positive (plus) or negative (minus). The distinction in each case was defined as two standard deviations above the mean of the binding of the 100 negative controls (typically 0.12 OD units).

Binding analysis. Analysis of antibody binding to H insulin was carried out in 3 representative sera: H insulin–specific IAA and non-H insulin–specific IAA and IA. Briefly, 500 μl of serum diluted 1:10 in phosphate-buffered saline (PBS) (pH 7.4) were incubated for 24 h at 20°C in the presence of 25,000 cpm of human A14 monoiodinated insulin purified by reverse HPLC (sp act 312 μCi/μg) (courtesy of Dr. B. Frank, Lilly), and a range of concentrations of unlabeled highly purified H insulin from 10^{-9} to 10^{-4} M. Each serum was also incubated in the absence of cold insulin to establish the maximum precipitable ^{125}I-labeled insulin. In addition, the analysis was carried out with normal H serum to assess background binding at each insulin concentration (2.5–3.4%). The labeled antibody complexes were precipitated with PEG 6000 (BDH, Poole, England) at 12.5% final dilution and washed three times with PEG. The resultant pellet was counted in an LKB Wallac γ-counter, and the data for each serum were analyzed with saturation binding curves (bound vs. log free) and Scatchard plots (bound/free ratio vs. bound insulin concentration) (9,10). Where applicable, affinities were calculated with a linear regression program.

Absorption studies. Human, P, and desalanated P insulins were covalently coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) (11) at a concentration of 2 mg insulin/ml beads. The binding of a limiting dilution of a representative serum from each group (H insulin–specific IAA and non-H insulin–specific IAA and IA) to different insulins was absorbed in a dose-dependent manner by preincubation of sera (16 h, 4°C at 1:30 final dilution) with different concentrations of Sepharose-coupled insulin (4×10^{-2} to 2×10^{4} μg insulin/ml serum). In each tube, the total mass of beads was kept constant by uncoupled CNBr-Sepharose 4B with ethanolamine-blocked active groups. Each serum was also preadsorbed with uncoupled beads alone to ascertain the maximum obtainable ELISA signal (designated 100%).

RESULTS

Binding to whole insulins. The selection of sera was such that 5 of the 10 autoimmune (IAA) sera (Nos. 6–10) bound all 3 insulins (H, P, and B), whereas the remainder (Nos. 1–5) appeared to be specific, or virtually specific, for H insulin. In contrast, the 10 diabetic (IA) sera (Nos. 11–20) bound all 3 insulins equally (Table 1).

Determinant restriction for whole insulins was investigated further by comparing the immunological cross-reactivity of the two groups of sera with rabbit, ovine, rat, and guinea pig insulins, which vary in 1, 4, 5, and 18 amino acid residues, respectively. The H insulin–specific IAA sera (Nos. 1–5) did not bind any of these variants, whereas all 5 non-H insulin–specific IAA sera (Nos. 6–10) bound rabbit and ovine insulin. Three (Nos. 8–10) also bound rat insulin and 2 (Nos. 8 and 9) bound guinea pig insulin. Similarly, although all 10 diabetic sera bound rabbit and ovine insulin, only 3 (Nos. 12, 15, and 16) cross-reacted with rat insulin and 2 (Nos. 13 and 15) cross-reacted with guinea pig insulin. Antibody binding to rat and guinea pig insulin in both autoimmune and diabetic sera, when detected, was at very low levels.

Binding to modified insulins. The H-specific IAA sera (Nos. 1–5) bound isolated H B-chain but bound neither P B-chain nor desalanated whole P insulin. In contrast, all the non-H insulin–specific IAA sera and all the IA sera bound desalanated P insulin but failed in all but 2 instances (Nos. 10 and 16) to bind H or P B-chain. Sera 10 and 16, which (unlike all others) bound P B-chain, also bound H B-chain and desalanated insulin.

Absorption studies. The binding to H insulin by an H insulin–specific IAA serum (No. 1) was extinguishable by preadsorption of the serum with H insulin coupled to Sepharose 4B, such that 33.5 μg insulin/ml serum produced 50% extinction (E_{50}) (Fig. 1*A*). However, binding was not reduced with P insulin–Sepharose, even at concentrations of 20,000 μg/ml serum. The binding of the same serum to isolated H insulin B-chain was also absorbed by H insulin–Sepharose (E_{50} = 40 μg/ml) but not by P insulin–Sepharose. The binding of representative non-H insulin–specific IAA serum (No. 7) to H insulin was absorbed by similar amounts of coupled H and P insulin (E_{50} = 1.8 and 3.2 μg/ml, respectively) (Fig. 1*B*). The E_{50} observed for its absorption by H insulin when binding P insulin was 5 μg/ml, and that observed for its absorption by P insulin when binding P insulin was 18 μg/ml. The representative IA serum (No. 11) was also unable to discriminate the two insulins and showed comparable E_{50} values in each case (6.7 and 25 μg/ml for binding to H insulin and 7.1 and 5.3 μg/ml for binding to P

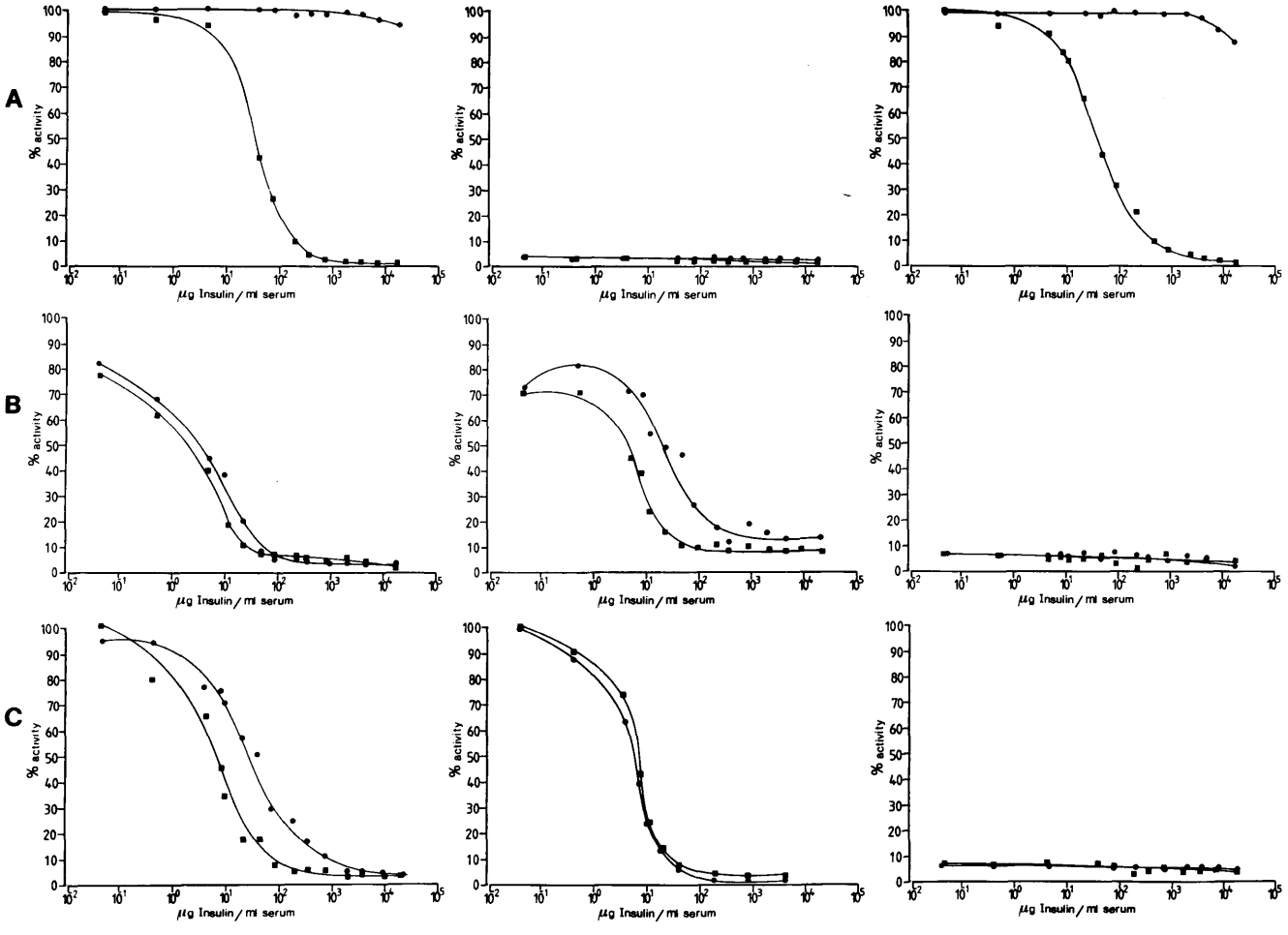

FIG. 1. Binding curves of human insulin–specific IAA serum No. 1 (*A*), non-human insulin–specific IAA serum No. 7 (*B*), and IA serum No. 11 (*C*) against human insulin (*left column*), porcine insulin (*middle column*), and human insulin B-chain (*right column*) after preadsorption with increasing amounts of porcine insulin (●) and human insulin (■) coupled to Sepharose 4B.

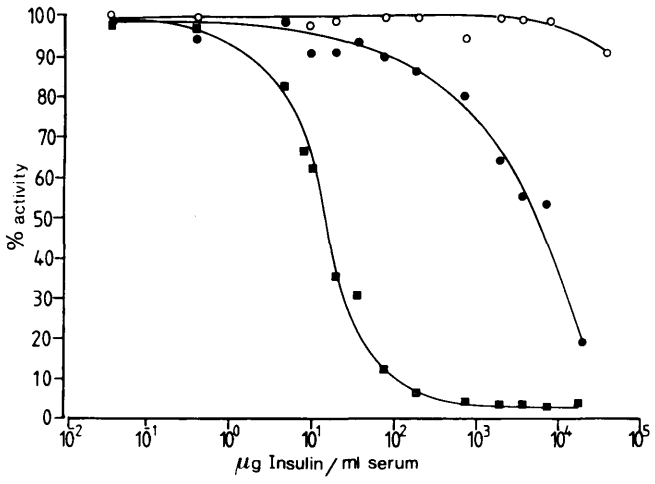

FIG. 2. Binding curve of human-specific IAA-containing serum No. 4 against human insulin after preadsorption with increasing amounts of human insulin (■), porcine insulin (●), and desalanated porcine insulin (○) coupled to Sepharose 4B.

insulin when adsorbed by H and P insulin–Sepharose, respectively) (Fig. 1*C*).

Serum No. 4 (IAA), classified as essentially H insulin specific on direct ELISA, nevertheless showed binding against P insulin that lay on the threshold of positivity. Its fine binding specificities were therefore examined more closely (Fig. 2). Preadsorption with coupled H insulin inhibited its binding to H insulin in the ELISA with an E_{50} of 14 μg/ml. E_{50} with coupled P insulin was 5000 μg/ml. No extinction, however, was obtained with coupled desalanated P insulin, even at concentrations of 20,000 μg/ml. Control experiments showed the coupled desalanated insulin capable of absorbing binding of a non-H insulin–specific IAA serum (No. 7) to H insulin with an E_{50} of 1.2 μg/ml to be comparable with the inhibition obtained with intact P insulin.

Reference serum specificity. The binding curves of the pooled reference serum against the different species of insulin is shown in Fig. 3. Binding to H, P, B, rabbit, and ovine insulins was similar, that to rat insulin was considerably less, and that to guinea pig insulin was barely detectable. The binding curves of the pooled reference (IA) serum and an H insulin–specific IAA serum (No. 1) to whole H insulin were nearly identical (Fig. 4*A*). When H insulin was replaced with isolated H insulin B-chain, however, the IA reference failed to react (Fig. 4*B*), and when it was replaced with desalanated P insulin, the IAA serum failed to bind (Fig. 4*C*). Finally, when the 2 sera (reference IA and H insulin–specific IAA) were mixed, being careful to maintain both at a dilution of 1:30, the binding curve against whole H insulin was displaced to the right, indicating an enhanced response; however, against porcine insulin, it remained unchanged (Fig. 4*D*).

Scatchard analyses. Scatchard plots of the binding analyses of a diabetic serum containing IA (No. 11), an H insulin–specific IAA containing serum (No. 1), and a non-H insulin–specific IAA containing serum (No. 7) are shown in Fig. 5. For each serum, the binding analysis was carried out over a range of concentrations wide enough to reach the inflection point of the plot of bound versus the logarithm of the free concentration, thus ensuring saturation had been reached. At each point, the nonspecific binding of a negative serum was measured and subtracted from the total bound. The Scatchard plot for the H insulin–specific IAA gave a straight line ($r = -.992$ based on 7 points) with an insulin-binding capacity of 2.25×10^{-7} M (33.3 U/L or 1.28 mg/L) and a binding affinity of 1.27×10^{7} L/mol. The plot for the IA serum was nonlinear, and the binding of ^{125}I by non-H insulin–specific IAA sera was insufficiently high to carry out a satisfactory Scatchard plot.

DISCUSSION

The insulin molecule comprises A- and B-chains consisting of 21 and 30 amino acid residues, respectively. The primary and tertiary structures of insulin are well known (12).

The direct and indirect binding profiles of individual IA and IAA sera to the insulin variants and fragments used in this study show convincing consistency. We observed distinct patterns of binding. H insulin–specific autoantibodies bound only whole H insulin and H insulin B-chain. Their dependence on a threonine residue at B30 was confirmed by their failure to bind whole P or B insulin, P B-chain (Ala-B30), or desalanated P insulin (B30 residue deleted). The E_{50} of the H insulin–specific IAA serum 4, which showed very low but perceptible levels of binding to P insulin, was measurable with P insulin, albeit 350 times greater than that observed with H insulin. Nevertheless, the absence of absorption with desalanated insulin would suggest that in H insulin–specific sera, even a minimal cross-reaction with P insulin is through a B30-restricted determinant.

All the IA-containing diabetic sera and 4 of the 5 non-H insulin–specific IAA sera bound exclusively A-chain deter-

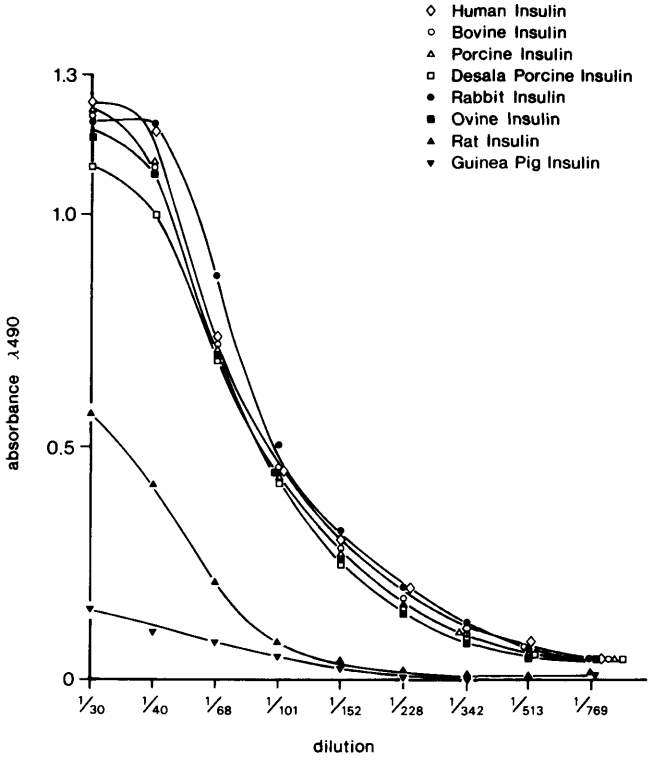

FIG. 3. Binding curves of polyclonal pooled reference (IA) serum against seven different mammalian insulins and a modified porcine insulin from which terminal B30 residue has been removed (desala porcine).

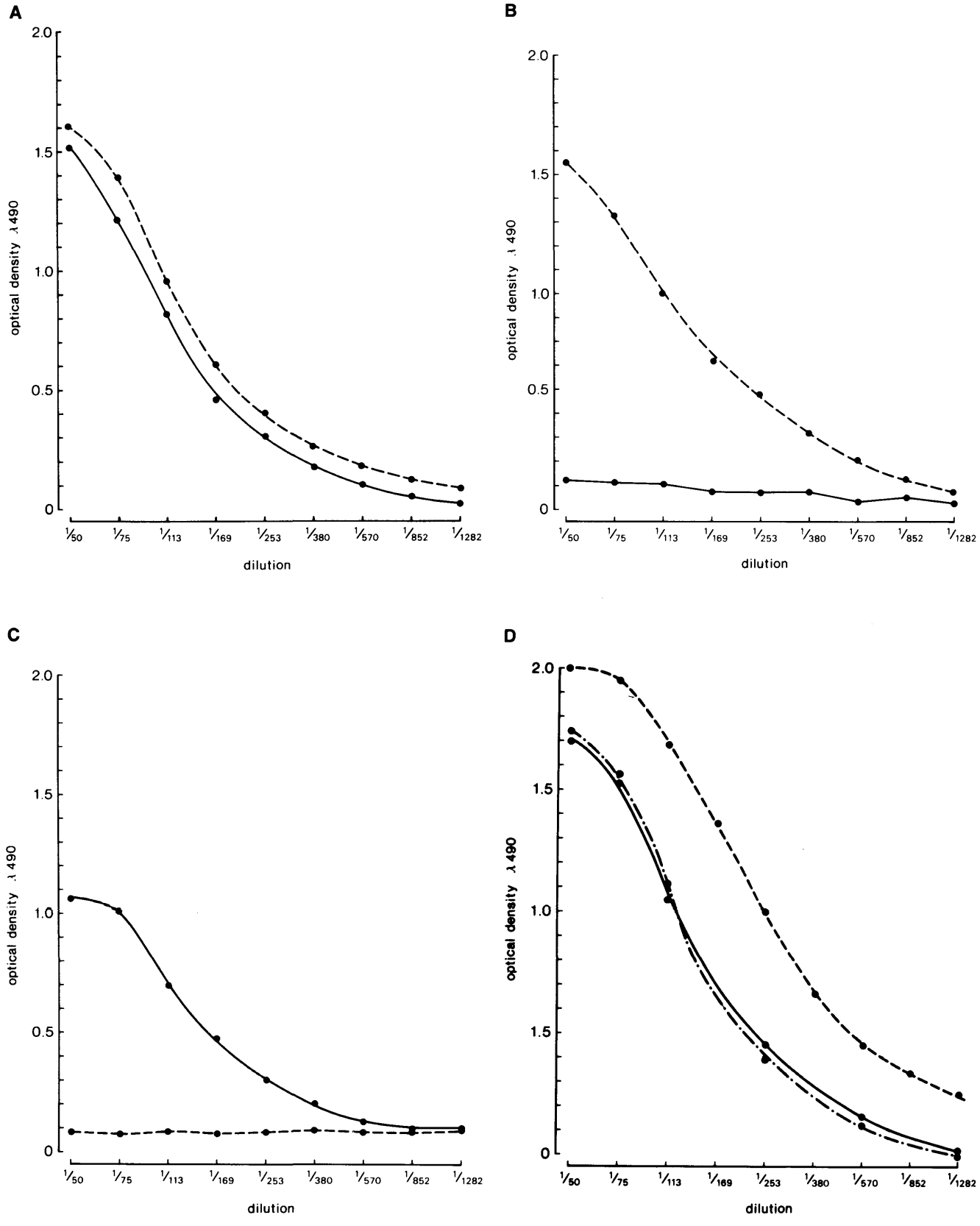

FIG. 4. Binding curves of reference IA serum (*solid lines*) and human insulin–specific IAA serum No. 1 (*dashed lines*) against whole human insulin (*A*), isolated human insulin B-chain (*B*), and desalanated porcine insulin (*C*). *D*: binding curve of reference IA serum against whole human insulin (*solid line*) and mixture of reference IA serum and human insulin–specific IAA No. 1 against whole human insulin (*dashed line*) and whole porcine insulin (*dashed-dotted line*).

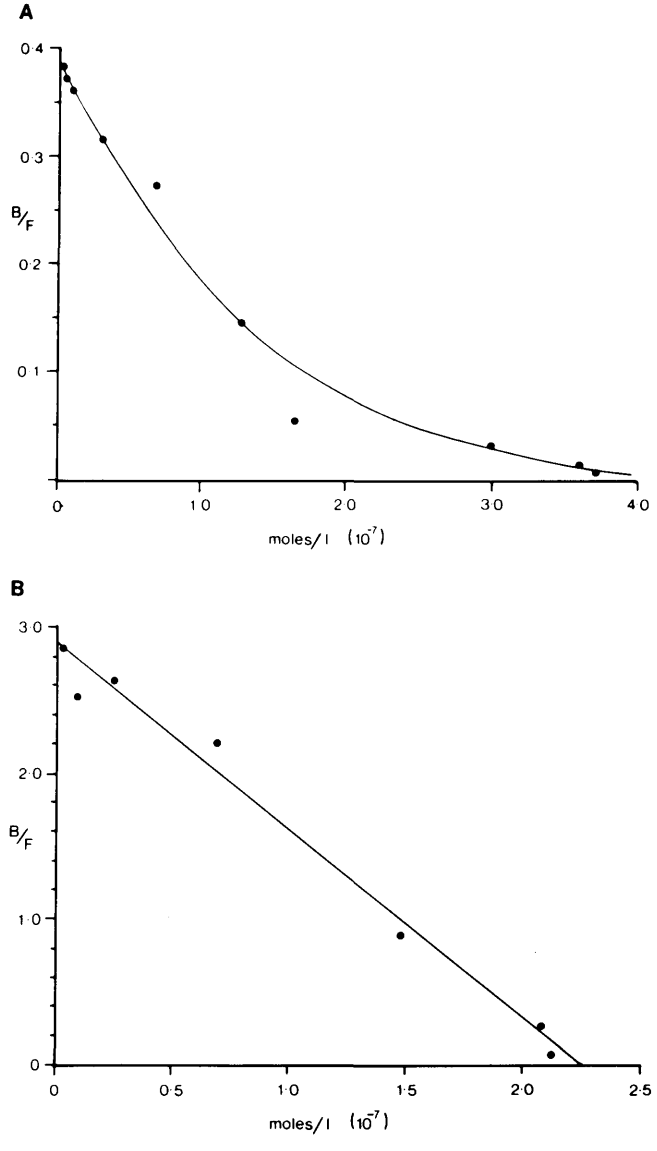

FIG. 5. Scatchard plots of binding curves of IA serum No. 11 (*A*) and human insulin–specific IAA serum No. 1 (*B*).

minants or determinants dependent on an intact tertiary structure, as demonstrated by the absence of binding to H and P B-chains. Isolated A-chain fragments cannot be used in such studies because the procedure for separation of the A- and B-chains causes reduction of the disulfide bond between A8 and A11 and changes the tertiary structure (5).

Serum No. 10 was unusual in that it contained non-H insulin–specific IAA capable of binding B-chain. These antibodies, however, were clearly not directed against Thr-B30 because the binding was almost as strong to P B-chain (0.51 OD units) as it was to H B-chain (0.64 OD units; negative control serum, 0.021 and 0.056 OD units, respectively) and desalanated P insulin (0.69 OD units). The possibility that cleavage of the whole molecule to obtain isolated P B-chains may have led to denaturation of the latter is unlikely in view of the observation that those sera that bound H but not P B-chain also bound whole H but not whole P insulin. The failure of serum No. 10 to bind rat and guinea pig insulins suggests that it may have been directed at a determinant involving B3 (Asn in H, P, B, ovine, and rabbit insulins, but Lys in rat insulin and Ser in guinea pig insulin), which is known to be antigenic (13–15). Rabbit insulin differs from H and P insulins by a serine-B30 substitution only, which confirms the patterns described above. Ovine insulin is similar to bovine insulin in structure and observed reaction.

We have remarked before that a proportion of IAA-containing sera in humans reacts exclusively with a species-defining (i.e., poorly conserved) epitope on H insulin (7), as if the evolutionary change in insulin structure had not, in some instances, been matched by the corresponding immunological adaptation. Tainer and colleagues (16) suggest that the antigenic sites on proteins are closely related to local mobility of the peptide backbone. The B30 residue has high mobility, and this may explain determinant restriction to Thr-B30 in sera Nos. 1–5; however, it does not account for the lack of B30 reactivity in sera Nos. 6–20. These latter sera (except for Nos. 10 and 16) were directed toward epitopes on the A-chain or conformational determinants. The A-chain displays two particular sites at A4 and A8–10 (α-loop) that along with B3 and B28–30 all occupy a band on the surface of the insulin molecule that shows high mobility (17). If mobility is an important factor in antigenicity, the A4 residue is a strong candidate for the reactions displayed by sera Nos. 6–9, 11–15, and 17–20: all the insulin variants used, except rat and guinea pig insulins to which binding was minimal, contain glutamic acid at A4.

Why binding to B30 and (probably) A4 should be mutually exclusive is unclear, although they are structurally close and possibly subject to mutual steric hindrance. The relative dominance of these sites for different subjects may then be determined by restriction in antigen presentation at the macrophage level or in the antibody repertoire and idiotype control. Note that the H-specific IAA represented 61% of 64 sequential IAA-positive sera, which we have identified in the context of polyautoimmunity. We have observed H insulin specificity only once in IAA from insulin-naive newly diagnosed diabetics, perhaps indicating a genetically determined polymorphism of antibody response to insulin. Any theory to explain this alternative dominance must also account for the apparent absence of B30-directed antibodies in diabetic sera. An important factor may be the availability or concentration of antigen. Endogenous insulin is released into the portal circulation as a dilute monomeric solution (18), whereas formulated insulins used in the treatment of diabetics are injected subcutaneously, where they polymerize and remain at a relatively high temperature for long periods before release into the systemic circulation (19). Polymerization greatly increases the antigenicity of insulin and may conceivably do so to the advantage of some determinants and the detriment of others. Thus, polymerization may mask the B30-dependent epitope.

The dilution curves in Fig. 4 confirm the main findings of this study and demonstrate clearly the extent to which IA and IAA differ in their epitope selection. The greater binding obtained when a mixture of H insulin–specific IAA and IA sera were reacted with H compared with P insulin indicated that B30-specific binding and A-chain binding were independent and summative.

Much debate has arisen over the value of Scatchard analysis and the misinterpretations that can result from binding ranges that are insufficiently extended or nonspecific binding that is incorrectly treated (10). We have paid close attention to these points and have found that the plot from an H-specific IAA serum was linear. This implies that the dynamics of the reaction could be described by a single class of binding site [with a binding capacity of 2.25×10^{-7} M (33.3 U/L) and a binding affinity of 1.27×10^{7} L/mol] and suggests that the serum was probably monoclonal for IAA. The calculated binding capacity is comparable with that given by Hirata and co-workers (20) (2×10^{-7} M) for patients with the insulin autoimmune syndrome. The plot for the IA-containing serum was nonlinear, suggesting polyclonality, and therefore could not be used to calculate binding constants.

The ELISA binding patterns and Scatchard studies reported in this study suggest that the antibody response to injected insulin is less restricted than the autoimmune response. This is perhaps due to the use in diabetics of heterologous insulins formulated with carriers such as protamine and Zn, which may result in considerable T- and B-lymphocyte activation. As a generality, H insulin–specific IAA in this study reacted with the B-chain and nonspecific IA and IAA reacted with the A-chain or with the three-dimensional determinants involving both chains. Isoelectric focusing experiments of these sera and further characterization of the non-H insulin–specific IAA are underway. For non-H insulin–specific IAA, this work should reveal whether the nonspecificity is related to a polyclonal response, as is the case for IA, or to a restricted response to a widely shared epitope.

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